The Role of HPV E6 Oncoprotein as a Biomarker in Anal Cancer Screening in Persons Living with HIV

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Abstract

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Early detection and prevention of anal cancer in high-risk groups such as persons living with HIV (PLWH) is of utmost importance. Most cases of anal cancer are caused by human papillomavirus (HPV), especially by HPV subtypes 16 and 18. Expression of E6 oncoprotein, particularly in HPV 16/18+ cases, plays a significant role in anal cancer oncogenesis. We evaluated the performance of HPV 16/18 E6 oncoprotein for detecting anal high-grade squamous intraepithelial lesions (HSIL). We analyzed clinical data collected over 125 visits from 82 PLWH (mean age 50.1 years; standard deviation 11.1 years) undergoing screening, high-resolution anoscopy (HRA) or treatment in Seattle, Washington, during 2015-2016. Demographic and clinical data, along with anal cancer screening, diagnosis, and treatment results, were collected through chart abstraction and electronic medical records review. Anal brush specimens were tested for type-specific high-risk HPV (hrHPV) DNA. Samples positive for HPV 16/18 were further tested for E6 oncoprotein. We described prevalence and

performance statistics of HPV16/18 E6 oncoprotein, HPV16/18 DNA and any hrHPV DNA as biomarkers. We calculated prevalence ratios (PR) for associations between these biomarkers and HSIL using a generalized linear model with a Poisson family and robust variance adjusted for CD4 count, HIV viral load and age. HPV 16/18 E6 was more specific but less sensitive than HPV16/18 DNA and any hrHPV DNA. HPV 16/18 E6+ showed 100% specificity, 100% positive predictive value and a statistically significant elevated adjusted PR of 6.23 (95% confidence interval: 1.12, 34.50) for HSIL compared to hrHPV negative samples; but demonstrated a low sensitivity (6.1%) and moderate negative predictive value (62.3%). Furthermore, we evaluated the HSIL disease extent data from HRA impression and found that 50% of four HSILs with >75% disease extent had E6+ samples, whereas none of the 30 HSILs with <25% disease extent had E6+ samples. Our study demonstrates that HPV 16/18 E6 oncoprotein is highly specific for identifying HSIL, but with low sensitivity, and may have utility for prioritizing lesions that might be at the highest risk for progression to cancer. Further investigation may establish E6 oncoprotein's role in early detection and prevention of anal cancer, either on its own or in combination with other biomarkers.

Introduction:

In the general population, anal cancer is rare, with an estimated 9,760 new cases and 1,870 mortalities for the year 2023 in the United States.[1] However, an alarming trend of rising incidence, advanced-stage presentation, and associated mortality has been observed in the United States and other developed nations since the 1970s.[2] Over the last decade, anal cancer incidence and mortality rates in the United States increased by approximately 2.2% and 2.9% annually, respectively.[3] The most frequent age group for the diagnosis of anal cancer is typically individuals between the ages of 55 and 64 years.[4] Even though the anal cancer incidence among the general population may be too low to warrant general population screening, certain subgroups exhibit significantly higher rates. These groups include persons living with human immunodeficiency virus (PLWH), men who have sex with men (MSM), women with a history of human papillomavirus (HPV)-related conditions, women living with HIV (WLWH) and immunocompromised populations unrelated to HIV.[5] Among these subgroups, MSM living with HIV face the highest risk of developing anal cancer, with incidence rates ranging from 77 to 137 cases per 100,000 individuals, surpassing the incidence rates of cervical cancer in some countries.[4, 6] HPV is an etiologic agent of anal cancer.[7] The prevalence of HPV DNA has been approximated at 94% in cases of anal high-grade squamous intraepithelial lesions (HSIL) and 88% in cases of anal cancer.[8] The increased prevalence of HPV infections in MSM living with HIV is considered a contributing factor to the excess burden of anal cancer within this population.[3]

Anal cancer comprises a range of malignancies, with anal canal tumors exhibiting pathological characteristics like that of squamous cell cervical cancer. Among 130 known types of HPV, 14

are high-risk HPV (hrHPV) types linked with cancer: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. Among them, HPV16 and 18 exhibit greater ability to circumvent the host's immune system and most anal cancers are attributed to HPV16.[3] The Lower Anogenital Squamous Terminology Standardization (LAST) project, focusing on HPV related lesions, introduced a two-tiered classification system for pre-invasive lesions in 2012, distinguishing low-grade squamous intraepithelial lesions (LSIL) from HSIL.[9] Within the anal canal, LSIL correlates with anal intraepithelial neoplasia (AIN)-1 or anal condylomata, while HSIL is associated with AIN-2 and AIN-3. This consensus-based system offers a nuanced understanding of the biological nature of these HPV-related lesions.[7] A meta-analysis revealed the transition risk from HSIL to cancer, indicating a pooled incidence of 45.9 per 100,000 men.[10] A nationwide cohort study in Denmark revealed that the risk of anal cancer increases with severity of the lesions, particularly among PLWH. The 5-year risk of anal cancer after AIN-3 is significantly higher in PLWH (14.1%) compared to those without HIV (3.2%).[11]

The Anal Cancer–HSIL Outcomes Research (ANCHOR) trial conducted from 2014- 2021, was designed with the aim of ascertaining whether treating anal HSIL is a safe and effective approach in reducing the likelihood of progression to anal cancer among individuals living with HIV, as compared to monitoring HSIL without administering treatment[12]. The ANCHOR trial found that among individuals who had biopsy-confirmed anal HSIL, the risk of developing anal cancer was notably reduced (by 57%) when they received treatment compared to those who underwent active monitoring. The findings from ANCHOR trial lend support to the adoption of screening and treatment for anal HSIL as the established standard of care for PLWH who are are≥35 years of age.

Current anal cancer screening recommendations include annual anal Pap testing for individuals ≥35 years of age among PLWH, MSM, or other high-risk groups. High-resolution anoscopy (HRA) and histology is recommended for LSIL or HSIL. HPV testing is recommended to triage patients with atypical squamous cells of undetermined significance (ASC-US)[13] but it is otherwise not recommended for anal cancer screening due to low specificity. A recent systematic review that evaluated the effectiveness of various tests, including anal cytology, anal hrHPV testing, p16 or p16/Ki-67 dual staining, and HPV E6/E7 messenger RNA (mRNA) testing, for identifying both precancerous and cancerous anal lesions found that HPV16/18 genotyping increases the overall specificity of hrHPV testing (with decreased sensitivity).[14] In particular, the pooled sensitivity and specificity of hrHPV testing for detecting AIN2 were 91.3% and 33.1% respectively, compared with 39.9% and 74.3% for HPV16/18 genotyping. Furthermore, HPV E6/E7 mRNA testing (pooled sensitivity: 74.3%, pooled specificity: 65.5), immunostaining for p16 or p16/Ki-67 dual staining (pooled sensitivity: 56.6%; and pooled specificity: 62.3%), and DNA methylation are also promising biomarkers for anal cancer screening and surveillance. In a study conducted by Siegel et al., an assessment of host DNA methylation in anal and cervical tissues from individuals with normal findings, HSIL, or cancer revealed the significant association of certain genes, such as ASCL1 and FMN2 (host viral markers), with the detection of HSIL.[15] A different study in PLWH also supported the connection between host methylation of ASCL1 and FMN2, and HSIL.[30] However, HPV E6/E7 oncoprotein has not been evaluated as a biomarker for anal HSIL.

The HPV E6 and E7 oncoproteins, which are generated by the HPV genes E6 and E7, have a significant role in the development of HPV-related cancer. They work by eluding the immune defenses of the host, disrupting the regulation of the cell cycle and apoptosis, and promoting the

accumulation of DNA damage and mutations.[16] In cervical carcinoma studies, researchers found elevated expression of E6 and E7 when HPV-infected squamous cells undergo precancerous or cancerous changes. This highlights the potential of testing for these viral oncoproteins as a more specific biomarker for precancer or cancer. A recently published systematic review and meta-analysis of 22 studies assessed the accuracy of an HPV E6/E7 oncoprotein test to identify cervical precancer and cancer.[19] Specificity ranged from 82.8%-99.1% across the entire population (irrespective of HPV status), as well as among hrHPV positive, HPV 16/18 positive and WLWH subpopulations, whereas sensitivity ranged from 54.2%-69.5%. Sensitivity improved moderately (60.8%-75.5%) in hrHPV positive or HPV16/18 positive women. The study also revealed that E6/E7 oncoprotein testing was more specific than hrHPV DNA testing, HPV 16/18 DNA testing, E6/E7 mRNA testing and cytology.

The goal of our study was to evaluate the performance characteristics of HPV 16/18 E6 oncoprotein for detecting prevalent anal HSIL as a biomarker in anal precancer and cancer screening of PLWH.

Methods:

Study Design, Study Setting & Population:

This study involved an examination of data based on samples collected during regularly scheduled clinical appointments for screening, HRA, or treatment attended by men living with HIV (MLWH) who are undergoing screening for anal cancer. These were done at the Harborview Medical Center Madison Clinic in Seattle, Washington, during the period spanning

September 2015 to December 2016. Eligible participants included individuals assigned male sex at birth, aged 18 and older, and living with HIV. Each participant could provide multiple samples during screening, HRA or treatment visits. Anal HPV testing was done at each visit as a part of this study. We evaluated the association between the detection of HPV 16/18 E6 oncoprotein and the presence of HSIL.

Participants recruitment strategy & enrollment:

The research coordinators examined the daily schedule of the Anal Dysplasia Clinic to identify potential participants. Recruitment occurred during clinical visits, and samples were collected at these visits. Recruitment efforts targeted both individuals with anal symptoms and those without, with the aim of encompassing a wide spectrum of patients seeking care at the clinic. The research coordinator approached potentially eligible patients during the clinic visit, explaining the study and confirming eligibility. Eligible participants provided written informed consent and HIPAA authorization prior to enrollment. Cash compensation of $10 was provided to participants for each visit.

IRB Approval:

This research project obtained ethical clearance from the institutional review board at the Fred Hutchinson Cancer Center.

Study Procedures:

Data Collection:

A certified anoscopist used an Anex® Brush (produced by Rovers Medical Devices, Oss, the Netherlands) to procure anal brush specimens from individual patients. One Rovers Anex brush specimen placed in a Hologic ThinPrep vial per patient per visit was utilized for HPV genotyping, detection of gene hypermethylation[30] and HPV 16/18 E6 oncoprotein testing for the study.

Supplementary information, age, race, ethnicity, HIV viral load, CD4 count, clinical findings from prior HRA exams, anal cytology, anal pathology, and preceding history of anal dysplasia were gathered through chart abstraction and electronic medical records (EMRs) review. CD4 count and HIV viral load details were extracted from the most recent laboratory measurement documented in the EMR. Race and ethnicity data were collected for mandatory National Institutes of Health (NIH) reporting and to characterize the patient population. Outcome data, sourced from clinical cytology and histology reports where available, were also collected. High-grade disease extent data were collected from clinical impression during HRA and confirmed with pathology where the percent disease extent is the maximum percent HSIL from both the intra-anal and perianal lesions.

At the conclusion of the study, a trained anoscopist conducted a thorough quality control review of medical records to assess disease extent and impressions for all HRA visits, including those up to and including the final study visit date.

Data Processing:

Anal specimens underwent processing at the HPV Research Group laboratory situated in the Harborview Research and Training Building in Seattle, Washington. DNA extraction from anal brush samples within ThinPrep® vials was carried out utilizing the QIAamp Blood DNA mini kit Subsequently, a portion of the extracted DNA underwent HPV genotyping through PCR, employing a Luminex liquid bead microarray assay.[17] This assay provided qualitative identification of 14 hrHPV types, specifically 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.

Specimens testing positive for HPV16 or HPV18 were also subjected to HPV-16/HPV-18 E6 oncoprotein testing using the OncoE6™ Anal Test, a prototype test developed by Arbor Vita Corporation (Fremont, CA), with results classified as positive or negative. The OncoE6™ Test utilizes an immunochromatographic approach with a lateral flow format. The test detects the E6 protein through a sandwich format employing high-affinity proprietary antibodies —specifically, a capture mAb / HPV E6 / detector mAb configuration. It is sensitive to <1 picogram for E6 detection / <1,000 cells per lateral flow strip and provides information on HPV 16/18 E6 oncoprotein through two distinct test lines. Additionally, a control line on each strip ensures the verification of detector reagent activity and proper migration of the sample solution up the test strip, as previously described.[18]

Exposure and Outcome Measurement:

To evaluate the role of HPV16/18 E6 oncoprotein as a potential biomarker in distinguishing between high-grade SIL and normal/low-grade SIL, the primary exposure we were interested in

was the detection of the E6 oncoprotein in participants with HPV16/18 infection. HPV16/18 positivity irrespective of E6 status and any hrHPV were also evaluated as exposures.

The primary outcome of interest was the grade of AIN based on anal SIL categorization. The SIL categorization was dichotomized to differentiate between no or low-grade lesions (NILM/LSIL) and pre-cancerous lesions (HSIL). This determination was based on available cytology and histology results obtained from clinical samples collected during the same visit. Specifically, we used a composite of cytology and histology test results. Cytology data were classified as HSIL when results indicated atypical squamous cells, HSIL cannot be excluded (ASC-H) or HSIL, and as NILM/LSIL when results indicated LSIL or lower. Histology data were categorized as HSIL when results indicated HSIL or AIN2-3 and as NILM/LSIL when results indicated AIN1 or lower. These categories align with current clinical management, triage, and risk-stratification practices. In cases where histology results were unavailable, cytology results were used to determine the outcome status, and vice versa. If both cytology and histology results were available and discordant, the more severe result was used to define the outcome. If neither cytology nor histology was performed, or if all available results were unsatisfactory, the study visit was excluded from the analysis.

The secondary outcome of interest was the disease extent of HSIL on HRA exam, specifically limited to cases of HSIL confirmed by cytology/histology. HRA impressions were abstracted by a single anoscopist. The percentage of HSIL disease was calculated based on the maximum of intra-anal and perianal lesions (e.g., 100% disease if circumferential intra-anal and perianal HSIL). Outcome groups were categorized as <25% HSIL, 25%-75% HSIL, and >75% HSIL.

Data Analysis:

We described the prevalence of HPV16/18 E6 oncoprotein+, HPV 16/18+ and any hrHPV+ in our sample and calculated their sensitivity and specificity for high-grade disease. We also calculated positive and negative predictive values for these biomarkers.

We calculated prevalence ratios (PRs) to evaluate the association between the exposure (categorized as HPV 16/18+ E6+, HPV 16/18+ E6-, positive for other [non-16/18] hrHPV only, or negative for hrHPV) and HSIL outcome. We opted for the PR as our measure of association considering the high prevalence of the disease (in this case, HSIL) in the study population. We utilized a generalized linear model (GLM) with a Poisson family and robust variance. Since we had correlated data due to multiple visits per subject, we used a GLM with an exchangeable correlation structure to generate PRs and 95% confidence intervals (CIs). Variables predefined as covariates consisted of CD4 count (categorized as low [<500 cells/µl]; high [≥500 cells/µl]; or unknown**;** latest available result), HIV viral load (categorized as detectable [≥40 copies/mL], undetectable [<40 copies/mL], or unknown; latest available result), and age (categorized as high [≥50years], low[<50years]; assessed at each visit). Crude and adjusted estimates, along with standard errors, were exponentiated to derive PRs and the corresponding 95% confidence intervals (CI).

We also evaluated disease extent data from the HRA impression and the HSIL disease extent to explore if those with E6 also have greater extent of the disease.

Statistical analyses were performed on R Statistical Software version RStudio 2023.09.1+494 "Desert Sunflower" with packages “sandwich” and “lmtest”.

Results:

Baseline characteristics of the study sample:

85 MLWH were enrolled for this study. Participants who returned for follow-up appointments during the study period had the opportunity to provide multiple specimens, resulting in a total of 133 specimens. Out of the 133 samples collected, eight (6%) were excluded from the analysis due to unsatisfactory cytology or histology data leaving 125 samples from 82 participants in the analysis. The mean age of the participants was 50.1 years (Standard Deviation 11.1 years) at the first visit (**Table 1**). Most of the participants self-identified as non-Hispanic (84.1%). The race distribution was mostly White (70.7%), followed by Black/African American (12.2%), Asian (4.9%) and American Indian/Alaskan Native (1.2%). At first visit, the mean CD4 count was 563.2 cells/µl with a standard deviation of 272.3 cells/µl. Additionally, 91.5% of participants exhibited an undetectable HIV viral load. Most participants (61.0%) contributed only one study visit, whereas 26.8% contributed to a second visit, 11.0% contributed a third visit and only 1.2% (a single participant) contributed a fourth visit.

HPV status and Anal dysplasia lesion grade:

Out of the 125 study samples, the majority (82.4%) had HPV detected. Among all samples, 38.4% were HPV 16/18 positive whereas 44.0% were positive for other [non-16/18] hrHPV only (**Table 2**). Three of the 48 HPV16/18 positive samples were also E6+ (**Table 2**). Two of the E6+ samples were contributed by one individual. Moreover, 39.2% had corresponding HSIL/AIN2-3, 49.6% had LSIL/AIN1 and 11.2% had NILM diagnosis according to cytology and/or histology data. Of the participants diagnosed with HSIL for whom HRA disease extent data was available

(N=45), the majority (66.7%) exhibited small lesions, constituting less than 25% of the anal canal or perianal region. Only 8.9% had circumferential (>75%-100%) intra-anal and perianal HSIL (**Table 3**).

Test performance characteristics for HSIL identification:

Our test performance evaluation shows sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for HSIL for three different biomarkers: Any hrHPV+, HPV 16/18+ (regardless of E6 status) and HPV 16/18 E6+. Among the three biomarker combinations, HPV 16/18 E6+ showed 100% specificity but low sensitivity (6.1%), and 100% PPV but moderate NPV (62.3%). HPV 16/18+ irrespective of E6 status showed lower specificity (75.0%) and PPV (60.4%), but higher sensitivity (59.2%) and NPV (74.0%). Any hrHPV+ irrespective of type or E6 status showed a low specificity (25.0%) and PPV (44.7%), but a high sensitivity (93.9%) and NPV (86.4%). Here the observed trend is that as we go from HPV 16/18 E6+ to all HPV 16/18+ to any hrHPV+, specificity and PPV decreases while sensitivity and NPV increases **(Table 4).**

Adjusted PRs for HSIL:

We constructed an adjusted model for calculating PRs for associations between the three HPV detection biomarkers and HSIL, using HPV negative as the referent group (**Table 5**). HPV 16/18 E6+ (adjusted PR 6.23; 95% CI: 1.12, 34.50) and HPV 16/18 E6- (adjusted PR 5.79; 95% CI: 1.38, 24.24) both demonstrated a statistically significant association with the detection of HSIL, while other [non-16/18] hrHPV+ only did not show a statistically significant elevated PR (adjusted PR 2.54; 95% CI: 0.58, 11.05), compared to the HPV negative referent group.

Descriptive analysis of HPV & HPV 16/18 E6 oncoprotein status and HRA disease extent:

The descriptive analysis of HPV detection & HPV 16/18 E6 oncoprotein status and HRA disease extent was limited to 45 visits with HSIL detection and non-missing disease extent data (**Table 6)**. Two of the 4 samples (50%) with corresponding >75% disease extent were HPV 16/18 E6 positive, whereas the other 2 were HPV16/18 E6 negative. None of the 30 samples with <25% disease extent was HPV16/18 E6+. **Supplemental table** details the HPV types detected, HPV 16/18 E6 status and disease extent for HSIL cases.

Discussion:

PLWH face the highest risk of anal cancer compared with persons living without HIV, emphasizing the need for improved screening and management practices to enhance early disease detection. To our knowledge, this is the first study to evaluate detection of E6 oncoprotein as a biomarker for anal HSIL. HSIL was present at nearly 40% of the study visits. HrHPV was very common (>80% of samples) but not specific for HSIL, whereas HPV 16/18 was more specific for HSIL (75%). HPV 16/18 E6 positivity was rare (3 samples representing 6% of HSIL visits) but was present only in those with HSIL (100% specificity). We also found that HSILs with greater disease extent were more likely to be E6 positive than HSILs with lesser disease extent.

The results of our study contribute to primary research on the detection of E6 oncoprotein as a biomarker for anal cancer screening. The inclusion of disease extent impressions obtained during HRA visits, along with high-grade cytology/histology results and HPV 16/18 E6 oncoprotein

status, allowed us to evaluate both the association of E6 positivity with anal HSIL and extent of HSIL disease. We found that E6 was associated with higher extent of the disease; 50% of four HSIL cases with >75% disease extent was E6 positive. However, none of the thirty samples with <25% disease extent was E6+. These disease extent findings provide support for the potential of E6 oncoprotein as a marker of more extensive or clinically relevant HSIL. Clinically, HSIL with higher disease extent are more likely to progress to oncogenesis and may be associated with higher recurrence rate after treatment.[20] The ANCHOR trial[12] showed that 43.3% of patients who developed anal cancer had >50% HSIL disease extent compared to 12.6% of patients who did not develop anal cancer.

While E6/E7 oncoprotein has not been evaluated as a biomarker for anal cancer screening, it has been evaluated as a biomarker for cervical cancer screening in multiple studies. A recently published systematic review and meta-analysis highlighted the performance characteristics of HPV E6/E7 oncoprotein tests as biomarkers for cervical cancer screening in women.[19] In pooled analyses of the population irrespective of HPV status, hrHPV+ women, HPV16/18+ women and WLWH, sensitivity ranged from 54.2% to 69.5% and specificity ranged from 82.8% to 99.1%. In particular, pooled sensitivity and specificity were 46.9% and 98.0% respectively in studies of WLWH. These results align with findings from various studies that have consistently shown stronger associations between E6 oncoprotein expression and the severity of cervical lesions compared to the correlation between HPV positivity and lesion severity.[18, 20] The authors noted that the considerable variation in performance across cervical studies may be attributed to factors such as the type of oncoprotein test (targeting E6, E7, or both proteins), whether it is a commercial or in-house developed test, the sample storage procedures, and the sample collection device. Specifically, accuracy estimates improved when samples were stored between −60 °C

and −80 °C and varied between studies using dry swabs or brushes with a liquid-based medium. In our study, sensitivity of E6 oncoprotein testing for HSIL was very low compared to the cervical studies, even when restricting to lesions with any hrHPV+ (3/46; 6.5%) or HPV16/18+ (3/29; 10.3%). Since in our study a brush placed into liquid medium was used and the samples were stored at ambient temperature (per the package insert), this is unlikely to be a cause of low sensitivity. It is however possible that low sensitivity may be due to low E6 oncoprotein quantity (i.e., below the level of assay detection) in some anal HSIL. Based on our finding that E6 positive samples tended to have greater disease extent, another possibility is that the E6 oncoprotein assay may specifically detect those HSIL that are more likely to progress to precancer or cancer.

While HRA-guided biopsy and histopathological evaluation is the current standard for surveillance of HIV-positive MSM due to their high PPV in detecting HSIL[21], no established management thresholds for anal cancer screening exists.[22] As clinicians need extensive training to attain proficiency in conducting HRA, only a limited number of providers have experience in HRA and can effectively identify high-grade lesions. The use of novel biomarkers, including E6, for predicting the malignant progression of AIN would significantly enhance patient management and quality of life outcomes.[23]

Other novel biomarkers, including but not limited to hrHPV DNA detection, HPV mRNA, immunostaining for p16 or p16/Ki-67 dual stain, and HPV or host viral gene DNA methylation, have been evaluated and may have a role in screening and management of pre-invasive anal lesions in PLWH.[24, 25] However, their performance for anal cancer varies across different populations.[26] HrHPV DNA detection as the primary screening method for anal cancer in high-

risk groups such as PLWH is constrained by the increased prevalence of anal hrHPV, although it has the highest sensitivity and lowest specificity when restricted to PLWH.[4, 27] Compared to that, HPV16/18 genotyping demonstrates significantly higher specificity, but its sensitivity is inadequate for a standalone test. Since elevated levels of p16/Ki-67 expressions are linked to greater severity of anal dysplasia these biomarkers may also help in characterizing anal precancerous lesions,[28] but their combined sensitivity and specificity for detecting anal precancer are only 56.6% and 62.3%.[29] Several studies that assessed the performance of E6/E7 mRNA in identifying anal precancer and cancer, both in HIV-positive and HIV-negative MSM, found that it offers higher specificity of 65.5% (95% CI, 58.5%-71.9%) but lower sensitivity of 74.3% (95% CI, 68.3%-79.6 when compared to HPV DNA testing.[24, 25] A separate study observed that the prevalence of DNA methylation biomarker positivity increased from AIN1 to cancer.[28] Another prior analysis utilizing the same data as ours compared host DNA methylation markers between HSIL and NILM/LSIL in PLWH.[30] Elevated levels of DNA methylation of ASCL1 and FMN2 were positively associated with HSIL and with HSIL extent, suggesting that host DNA methylation testing holds promise as a screening and triage tool for anal HSIL as a biomarker in anal precancer and cancer screening of PLWH. As such there are currently no optimal screening modalities for anal cancer that exhibit both high sensitivity and specificity, and it remains to be seen whether combining biomarkers increases test performance.[4] Given the high specificity but low sensitivity of E6 oncoprotein for anal HSIL in our study, there could be potential for using combinations of E6 oncoprotein and other novel biomarkers for anal cancer screening and cancer risk stratification, possibly charting a pathway for their inclusion in future anal cancer screening guidelines.

The scope of this study, as well as the conclusions drawn from it, are constrained by several factors. First, our sample size is relatively modest, comprising a total of 125 samples collected from 82 individuals, and statistical power limited by the small number of samples with HPV16/18+ E6+ oncoprotein results: ~2% of the clinical visits (3 out of 125) involving ~2% of the patients (2 out of 85) had HPV16/18+ E6+ detection. In a future expanded study, we would aim to increase the total number of study participants including individuals belonging to other at-risk populations who might develop HSIL. Second, because of the limited amount of follow up, we were not able to assess these factors in a longitudinal way. Finally, not all participants underwent confirmatory histology, and we relied on a composite outcome derived from the existing data from medical records for histology and cytology.

PLWH are at an elevated risk of anal HPV infection and the progression to HSIL and anal cancer. These lesions pose therapeutic challenges, and surgical intervention often entails significant postoperative complications.[31] An inherent challenge in studies of HSIL is that it is unknown which lesions would develop into cancer. The ANCHOR trial provided evidence that treatment of HSIL is effective for preventing anal cancer. However, given that HRA is resource intensive (due to high cost and the need for highly trained anoscopists), there may be value in utilizing biomarkers that could identify individuals at highest risk for progression. In conclusion, this study demonstrates a high specificity and PPV for oncoprotein testing in individuals diagnosed with HSIL, accompanied by low sensitivity and moderate NPV estimates. In future studies, we need to evaluate whether there are ways to increase sensitivity of the E6 oncoprotein test (either on its own or by combining with other biomarkers). Moreover, since it is a highly specific for identifying HSIL and seems to be more likely to identify lesions with greater disease extent, there could be utility in situations where there is limited access to HRA for identifying

lesions that might be at highest risk for progression. Further investigation is necessary to establish whether highly specific E6 tests may have a role in early detection and prevention of invasive cancer development in conjunction with other biomarkers.

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Table 1: Baseline Characteristics of persons living with HIV, attending the anal dysplasia clinic, included in the study (N= 82 persons)[1]

Characteristics	N (%)
Mean age (years) ± SD	50.1 ± 11.1
Ethnicity	
Hispanic	10 (12.2%)
Non-Hispanic	69 (84.1%)
Unknown	3 (3.7%)
Race	
American Indian/Alaska Native	1 (1.2%)
Asian	4 (4.9%)
Black/ African American	10 (12.2%)
White	58 (70.7%)
Unknown	9 (11.0%)
Mean CD4 count (cells/µl) ± SD	
CD4 recent numbers	563.2 ±272.3
HIV viral load[2]	
Undetectable	75 (91.5%)
Detectable	5 (6.1%)
Unknown	2 (2.4%)
Number of HRA visits per participants[3]	
1	50 (61.0%)
2	22 (26.8%)
3	9 (11.0%)
4	1 (1.2%)

[1] 82 persons out of 85 had corresponding cytology/histology data.
[2] Viral load is categorized as undetectable for individuals with <40 copies/mL and detectable otherwise per clinical guideline.
[3] Number of HRA visits per participant could include first screening, follow-up, or treatment visits.

Abbreviations: Standard deviation (SD); High-resolution anoscopy (HRA)

Table 2: HPV DNA and HPV16/18 E6 oncoprotein results (N=125 visits)

HPV status (N=125 visits)[1]	**N (%)**
Any hrHPV types detected	103 (82.4%)
HPV 16/18 detected	48 (38.4%)
HPV16/18 E6+	3 (6.3%)[2]
HPV16/18 E6-	45 (93.8%)
HPV 16/18 not detected (other hrHPV types only)[3]	55 (44.0%)
No HPV detected	22 (17.6%)

[1] 125 visits out of 133 had corresponding cytology/histology data.
[2] Here the denominator is 48. 3 specimens out of 48 were positive for HPV16/18 E6.
[3] Other hrHPV subtypes were 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.

Abbreviations: Human papillomavirus (HPV); high-risk HPV (hrHPV)

Table 3: Anal dysplasia lesion grade according to cytology/histology data[1] (N=125 visits)[2]

HPV status (N=125 visits)	N (%)
NILM	14 (11.2%)
LSIL/AIN1	62 (49.6%)
HSIL/AIN2-3	49 (39.2%)
HSIL disease extent[3]: (N=45)[4]	
<25%	30 (66.7%)
25%-75%	11 (24.4%)
>75%-100%	4 (8.9%)

[1] Combined cytology/histology result
[2] No specimen for the remaining 8. Cytology/histology data is available for 125 study visits.
[3] High-grade disease extent was collected from clinical impression during HRA and confirmed with pathology; the percent disease extent is the maximum percent HSIL from both the intra-anal and perianal lesions.
[4] 49 out of 125 visits resulted in HSIL. 45 out of 49 visits have corresponding HRA disease extent data.

Abbreviations: Human papillomavirus (HPV); High-grade squamous intraepithelial lesion (HSIL); Low-grade squamous intraepithelial lesion (LSIL); Anal intraepithelial neoplasia, grades 1-3 (AIN1-3); Negative for intraepithelial lesion or malignancy (NILM)

Table 4: Test performance characteristics for HSIL Identification (N=125 visits)[1]

	HSIL/AIN 2-3 (N=49)	LSIL/AIN 1 & NILM (N=76)	Sensitivity for HSIL	Specificity for HSIL	PPV	NPV
HPV 16/18 E6+	3	0	6.1%	100.0%	100.0%	62.3%
HPV 16/18+	29	19	59.2%	75.0%	60.4%	74.0%
Any hrHPV+	46	57	93.9%	25.0%	44.7%	86.4%
HPV negative	3	19	-	-	-	-

[1] Cytology/histology data available for 125 study visits.

Abbreviations: Human papillomavirus (HPV); high-risk HPV (hrHPV); High-grade squamous intraepithelial lesion (HSIL); Low-grade squamous intraepithelial lesion (LSIL); Anal intraepithelial neoplasia, grades 1-3 (AIN1-3); Negative for intraepithelial lesion or malignancy (NILM)

Table 5: Unadjusted and adjusted prevalence ratios (PRs) for HSIL using GLM[1] with family Poisson and robust standard error (N=125 visits)[2]

HPV & HPV 16/18 E6 Oncoprotein Status	HSIL		Total	PR Unadjusted	95% CI	PR Adjusted[3]	95% CI
	Yes N=49	No N=76					
HPV 16/18 E6+	3	0	3	7.33	(2.44, 22.07)	6.23	(1.12, 34.50)
HPV 16/18 E6-	26	19	45	4.24	(1.37, 13.13)	5.79	(1.38, 24.24)
Other hrHPV+ *only*	17	38	55	2.27	(0.70, 7.32)	2.54	(0.58, 11.05)
HPV negative	3	19	22	1		1	

[1] Using Generalized Linear Modelling for data analysis
[2] cytology/histology data available for 125 study visits.
[3] Adjusted for age, CD4 count and Viral load.

Abbreviations: Human papillomavirus (HPV); high-risk HPV (hrHPV); High-grade squamous intraepithelial lesion (HSIL); Prevalence ratio (PR); Confidence interval (CI); Generalized linear model (GLM)

Table 6: Descriptive analysis of HPV & HPV 16/18 E6 Oncoprotein status and HRA disease extent among those with HSIL: (N=45 visits)[1]

	HSIL disease extent					
HPV & Oncoprotein E6 Status	**<25% (N=30)**		**25-75% (N=11)**		**>75-100% (N=4)**	
HPV 16/18 E6+	0	0.0%	1	9.1%	2	50.0%
HPV 16/18 E6-	17	56.7%	6	54.5%	2	50.0%
Other hrHPV+ *only*	11	36.7%	3	27.2%	0	0.0%
HPV negative	2	13.3%	1	9.1%	0	0.0%

[1] 49 visits resulted in positive HSIL detection. Among them only 45 had disease extent results.

Abbreviations: Human papillomavirus (HPV); high-risk HPV (hrHPV); High-grade squamous intraepithelial lesion (HSIL); High-resolution anoscopy (HRA)

Supplementary Table: HPV subtypes detected with HRA HSIL disease extent of N=45[1] (Table sorted in descending order according to persons with highest disease extent)

Study Id-Visit number	HPV Types Detected	HRA disease extent
10-1	HPV 18 **(E6 -)**, 33, 51, 59	>75%
18-1	HPV 16 **(E6 +)**, 31, 52	25% to 75%
18-2	HPV 16 **(E6 +)**, 31, 52	>75%
23-1	HPV 16,18 (**E6** -), 31, 39, 52	>75%
24-1	HPV 16 **(E6 +)**, 31, 33, 52	>75%
1-1	HPV 31 & 68	25% to 75%
5-1	HPV 16 **(E6 -)**, 18, 45, 56	25% to 75%
11-4	HPV 16 **(E6 -)**, 39, 52	25% to 75%
12-1	HPV 59, 68	25% to 75%
13-1	*No HPV*	25% to 75%
14-1	HPV 16, 18 **(E6 -)**, 33	25% to 75%
26-1	HPV 31, 33, 35, 51	25% to 75%
27-1	HPV 16 **(E6 -)**	25% to 75%
28-1	HPV 16 **(E6 -)**	25% to 75%
30-2	HPV 16 **(E6 -)**, 31, 52, 66	25% to 75%
2-1	*No HPV*	<25%
2-2	*No HPV*	<25%
3-1	HPV 31 & 35	<25%
3-2	HPV 31 & 35	<25%
3-3	HPV 31	<25%
4-1	HPV 51	<25%
6-1	HPV 16 **(E6 -)**	<25%
6-2	HPV 16 **(E6 -)**	<25%
7-1	HPV 16, 18 **(E6 -)**, 31, 33, 39. 45 & 52	<25%
8-1	HPV 16 **(E6 -)**, 31, 56	<25%
9-1	HPV 18 **(E6 -)**, 31, 68	<25%
11-1	HPV 16 **(E6 -)**, 39, 56	<25%
11-2	HPV 16 **(E6 -)**, 39, 51, 56	<25%

[1] 49 visits resulted in positive HSIL detection. Among them only 45 had disease extent results.

15-1	HPV 39, 51, 52	<25%
17-1	HPV 52	<25%
19-1	HPV 16 **(E6 -)**, 31, 58	<25%
20-1	HPV 31, 58	<25%
21-1	HPV 16, 18 **(E6 -)**, 45, 66	<25%
21-2	HPV 16, 18 **(E6 -)**, 45, 66	<25%
22-1	HPV 56, 66	<25%
25-1	HPV 51	<25%
27-3	HPV 16 **(E6 -),** 31	<25%
29-1	HPV 16 **(E6 -)**, 45	<25%
30-1	HPV 16 **(E6 -)**, 52, 66	<25%
31-1	HPV 51	<25%
32-2	HPV 16 **(E6 -)**, 39, 58, 59	<25%
33-1	HPV 45, 51, 59	<25%
34-1	HPV 16 **(E6 -)**, 33	<25%
35-1	HPV 16, 18 **(E6 -)**, 33, 45	<25%
36-1	HPV 16 **(E6 -)**	<25%

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